UNIVERSITI PUTRA MALAYSIA

AN EXAMINATION OF GENE EXPRESSION (ACC OXIDASE AND RECEPTOR-LIKE PROTEIN KINASES) IN SOMATIC EMBRYOGENESIS OF OIL PALM (ELAEIS GUINEENSIS)

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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In plants, complete embryos can develop not only from the zygote, but also from somatic cells. This biological process is known as ' somatic embryogenesis'. Plant regeneration *via* somatic embryogenesis has provided the means for the application of biotechnology techniques such as clonal propagation, genetic transformation and cryopreservation. The significant potential of this *in vitro* propagation has resulted in a spate of exploration in a wide range of plants particularly commodity crops.

In Malaysia, somatic embryogenesis of oil palm (*Elaeis guineensis* Jacq.) has generated a great deal of research interest. Although the totipotency of somatic cells has been documented in oil palm (Schwendiman *et al.*, 1990; Duval *et al.*, 1995; Wong *et al.*, 1999), the mechanism of how the somatic cells undergo the change in fate to become embryogenic remains largely unknown.

The understanding of this fundamental process is vital for the development of an economically viable propagation system. Hence, this study was conducted to explore the molecular events of oil palm somatic embryogenesis to enable isolation of developmental stage-specific markers that will greatly facilitate the improvement of the *in vitro* system. Two different approaches were carried out to identify these genes.

In the first approach, specific genes from the multigene family of protein kinases were isolated and their expression profiles were examined during oil palm somatic embryogenesis. Three receptor-like protein kinases (RLKs) designated as D4.5, E8.1.1 and F3.1 were characterised in this study. Expression studies and sequence analysis have revealed plausible roles of D4.5 and F3.1 as components of the plant growth regulator induced signalling pathway, meanwhile E8.1.1 most probably plays a role in mediating disease-resistance.

The second approach was to use the strategy of suppression subtractive hybridisation (SSH), subtracting cell-suspension culture cDNAs from the embryoid cDNAs in order to obtain embryo-enhanced-transcripts that are differentially regulated

during the transition of embryogenic competent callus to the mature embryogenic stage. A total number of 200 clones (suspension as tester) and 69 clones (embryoid as tester) from the forward and reverse subtraction cDNA libraries, respectively, were randomly isolated and preliminary analysed by reverse Northern. Sequence analysis of 30 clones revealed that a large proportion of the genes, 57% were of unknown function while the remaining 43% were related to various biological pathways.

Four SSH clones: 269-10-D2 (ACC oxidase), 269-112-a2 (protein-disulphide isomerase like), 269-155-A5 (6-phosphogluconolactonase-like) and 2524-4-b1 (no significant similarity) were selected for further characterisation. The transcripts of these clones were found to accumulate only in embryogenic tissues. Although their expression patterns signify a potential as an embryogenic marker, preliminary Northern analysis is insufficient to draw a clear conclusion about their role in embryogenesis.

To further investigate the expression of the 269-10-D2 gene, the accumulation of 269-10-D2 transcripts were examined in different cell-lines of cell-suspension, embryoid and non-embryogenic cultures. The results indicated that the level of 269-10-D2 expressions was clonal-dependent and was not affected by different hormone treatments. *In situ* RNA hybridisation in suspension culture sections from various cell lines demonstrated that the 269-10-D2 gene was expressed in the protodermis-like layer. This result suggests that the accumulation of 269-10-D2 could be correlated with early events of the somatic embryogenesis process. Thus, 269-10-D2 may serve as a useful molecular marker for early somatic embryo development.

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Di dalam tumbuhan, embrio yang lengkap bukan sahaja berkembang melalui zigot tetapi juga daripada sel somatik. Proses biologikal ini dikenali sebagai 'embriogenesis somatik'. Pertumbuhan semula tumbuhan melalui embriogenesis somatik memberi makna kepada aplikasi teknik bioteknologi seperti pembiakan klonal, transformasi genetik dan pengawetan sejuk. Potensi pembiakan *in vitro* yang berkesan ini telah memulakan detik penerokaan eksplorasi kajian embriogenesis somatik ke atas pelbagai jenis tumbuhan terutamanya tumbuhan komoditi.

Di Malaysia, embriogenesis somatik kelapa sawit (*Elaeis guineensis* Jacq) telah menjanakan minat terhadap penyelidikan. Walaupun totipotensi sel somatik kelapa sawit telah didokumentasikan (Schwendiman *et al.*, 1990; Duval *et al.*, 1995; Wong *et al.*, 1999), kebanyakan mekanisme perubahan takdir sel somatik kepada sifat embriogenik masih tidak diketahui.

Pemahaman asas proses ini adalah penting untuk perkembangan sistem pembiakan yang dapat wujud secara ekonomi. Maka, kajian ini dilaksanakan untuk menerajui kejadian molekular embrio somatik kelapa sawit untuk mengasingkan petanda yang specifik terhadap tahap perkembangan yang akan membantu dalam peningkatan sistem *in vitro* ini. Dua cara yang berlainan telah dilaksanakan untuk mengenalpasti gen-gen ini.

Dalam cara pertama, gen-gen spesifik daripada gen pelbagai keluarga 'protein kinase' telah dipencilkan dan ekspresi mereka telah dikaji di dalam proses embiogenesis somatik kelapa sawit. Tiga ' receptor-like protein kinase' (RLK) yang dikenali sebagai D4.5, E8.1.1 dan F3.1 telah dicirikan di dalam kajian ini. Kajian ekspresan gen dan analisa penjujukan telah menunjukkan kemungkinan D4.5 dan F3.1 memainkan peranan sebagai komponen yang terlibat dalam isyarat laluan yang diaruh oleh pengawalatur pertumbuhan tumbuhan, sementara E8.1.1 berkemungkinan besar memainkan peranan sebagai perantara dalam pelaksanaan rintagan terhadap penyakit.

Cara kedua menggunakan strategi ' Suppression Subtractive Hybridisation' (SSH), yang menyingkirkan cDNA sel kultur ' suspension' daripada cDNA embriod untuk menghasilkan transkrip yang merangsangkan perubahan sel kultur 'suspension' ke tahap embrio yang matang. Sejumlah 200 klon (dengan 'suspension' sebagai 'tester') dan 69 klon (dengan embriod sebagai 'tester') yang dihasilkan daripada penyingkiran ke hadapan dan ke belakang masing-masing, telah dipencilkan secara rawak dan analisa 'reverse Northern' telah dijalankan. Analisa penjujukan daripada 30 klon telah menunjukkan sebahagian besar daripada gen-gen (57%) tidak diketahui peranannya manakala 43% telah dikaitkan dengan pelbagai tapak jalan biologikal.

Empat klon SSH: 269-10-D2 (ACC oxidase), 269-112-a2 (protein-disulphide isomerase like), 269-155-A5 (6-phosphogluconolactonase-like) dan 2524-4-b1 (tiada persamaan nyata) telah dipilih untuk pencirian selanjutnya. Trankrip klon-klon ini telah ditemui di tisu embriogenik sahaja. Walaupun corak pengekspresan mereka melambangkan potensi sebagai petanda embriogenik, kajian awal analisa 'Northern' adalah tidak memadai untuk membuat keputusan yang muktamad terhadap peranan mereka dalam proses embriogenesis.

Untuk melanjutkan penyiasatan ekspresi gen 269-10-D2, pengumpulan transkrip 269-10-D2 telah disiasat ke atas sel 'suspension', embriod dan tisu bukan embriogenik yang mempunyai sel selanjar yang berlainan. Keputusan menunjukkan bahawa paras ekspresi 269-10-D2 adalah bergantung kepada 'clonal' dan tidak dipengaruhi oleh rawatan hormon. Hybridasi RNA secara *in situ* yang dilakukan pada keratan-keratan sel

'suspension' yang mempunyai sel selanjar yang berbeza telah menunjukkan ekspresi 269-10-D2 di bahagian lapisan seperti protodermis. Keputusan ini mencadangkan kehadiran 269-10-D2 itu berkait dengan kejadian awal proses embriogenesis somatik. Maka, 269-10-D2 amat berguna sebagai petanda molekular perkembangan awal embrio somatik.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

α	alpha
β	beta
λ	lambda
%	percentage
°C	degree centigrade
2-BE	ethyleneglycolmonobutylether
bp	base pair
BLAST	Basic Local Alignment Search Tool
BSA	bovine serum albumin
Ci	curie
C-terminal	carboxyl terminal
2,4-D	2,4-dichlorophenoxy acetic acid
DNA	deoxyribonucleic acid
Dnase I	deoxyribonuclease 1
cDNA	complementary DNA
dNTPs	deoxynucleotides
dATP	2'-deoxy-adenosine-5'-triphosphate
dCTP	2'-deoxy-cytidine-5'-triphosphate
dGTP	2'-deoxy-adenosine-5'-triphosphate
dTTP	thymidine-5'-triphosphate
dH_2O	distilled water
DEPC	diethyl pyrocarbonate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol bis- (β-aminoethyle ether)
EM	embryoid
EtBr	ethidium bromide
g	gram

HCl	hydrochloric acid
hr	hour
Jacq.	Jacquin
LB	luria-bertani
kb	kilobase
L	liter
LiCl	lithium chloride
LRR	leucine-rich repeats
M	molar
mg	milligram
min	minute
ml	millimetre
mm	millimetre
mM	millimolar
mmol	millimole
MMLV	Maurine Moloney Leukemia Virus
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
MOPS	3-(N-morpholino) propane-sulphonic acid
mRNA	messenger RNA
NaCl	sodium chloride
NaOAc	sodium acetate
NE	non-embryogenic
ng	nanogram
N-terminal	amino terminal
OD	optical density
PCR	polymerase chain reaction
pfu	plaque forming unit
Poly A+RNA	polyadenylated RNA
PVP	polyvinylpyrrolidone
PVPP	polyvinylpolypyrrolidone

CHAPTER 1

INTRODUCTION

Embryogenesis is a fundamental biological process in the plant life cycle. In the most highly evolved class of plants, known as angiosperms (Angiospermae) or flowering plants, embryogenesis involves an array of developmental episodes beginning with a single-celled progenitor, the zygote, and ending with the formation of a mature embryo. Interestingly, embryogenesis in plants can commence from cells other than the fertilised egg cell. In contrast to other eukaryotes, the differentiation programme in plants is flexible, as almost any fully differentiated plant cell can become embryogenic under defined conditions.

One of the asexual embryo formation mechanisms is known as 'somatic embryogenesis'. This is a process by which somatic cells develop through the stages of embryogeny into a whole plant without gametic fusion. The generation of a totipotent state in somatic cells is indeed a remarkable biological phenomenon. Since the first observation of somatic embryo formation in carrot cell suspension cultures by Steward *et al.* (1958), the potential for asexual embryogenesis has been investigated in a wide range of plant species.

The process of somatic embryogenesis can be initiated by simply manipulating the auxin content in the growth medium, which will result in adequate quantities of synchronously staged embryos. This type of vegetative propagation would be an undoubted advantage in the areas of fundamental research as well as breeding

programmes for many types of plants whose only natural reproduction is sexual. This is the case for the African oil palm, *Elaeis guineensis*, a true allogamous species for which conventional methods of vegetative propagation cannot be applied. However, the technology of plant tissue culture in monocots lapses behind the dicots. Monocotyledoneous plants were once thought to be recalcitrant in *in vitro* systems (Stirn *et al*., 1995). Due to this ingrained feeling, researchers were reluctant to invest in experiments for which conclusions were foregone.

However, in view of the economic importance of oil palm today, it was inevitable that the potential for somatic embryogenesis in this palm be explored. The potential advantages of somatic embryogenesis in oil palm *E. guineensis* for breeding purposes and its application to synthetic seed technology have been widely investigated (Besse *et al*., 1992; Duval *et al*., 1995; Morcillo *et al*., 1999). Currently, in Malaysia there are at least 10 companies/organisations that have been extensively producing elite palms for field evaluation of clonal performance and fidelity (Wong *et al*., 1999). Although *in vitro* propagation of oil palm *via* somatic embryogenesis has been attained, somaclonal variations (Jaligot *et al*., 2000), poor rates of plantlets regeneration (Morcillo *et al*., 1999) and flower abnormalities were some of the problems encountered. Thus, in order to improve rates of somatic embryo propagation, maturation and subsequent regeneration, the biological process that underlies somatic embryogenesis must first be understood.

Plant embryogenesis is a particular complex process. The most promising method for identifying the mechanisms responsible for the key events of embryogenesis will

come from molecular and genetic analysis. Much of the available information on somatic embryogenesis is not devoted to studies on gene expression. In order to understand the biological processes that govern plant embryo formation, genes that contribute to the embryo programme must first be identified and then studied in detail.

In this study, different approaches were carried out to identify these genes. The first approach was to target known genes, which have been reported to be involved in embryogenesis. The multigene family protein kinases were selected in this study due to their well known signalling function in plant development. The second approach was to use the strategy of subtraction hybridisation, subtracting cell-suspension culture cDNA from the embryoid cDNA in order to obtain embryo enhanced-transcripts that are expressed during the transition of embryogenic competent callus to the mature somatic embryo. This study of gene expression during plant embryogenesis is focused on identifying molecular markers from somatic embryos and characterising the expression and regulation of these genes through embryo development, which will eventually enable the unravelling of the complex regulatory network controlling embryogenesis.